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Frequency of Hepatitis B Virus e-Minus Mutants Varies Among Patients From Different Areas of China

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Four hundred forty-six serum samples from HBsAg–positive chronic hepatitis B patients were collected from five areas in China (eastern coastal city, Shanghai; southwestern inland city, Chengdu; mid-inland city, Wuhan; southern island city, Haikou; and northeastern city, Changchun). Precore stop codon variants (e-minus mutants) were screened using a rapid method of polymerase chain reaction (PCR) amplification of a precore and partial core gene fragment (nucleotides 1785–2172), followed by dot-blot hybridization with specific oligonucleotide probes (M0, and M1 + M2). The sequence of the M0 probe covered the distal precore region of wild-type virus (nucleotides 1887–1908), and the sequences of the M1 and M2 probes were from sequences mutated at nt. 1898, (TGG r TAG) with or without additional change at nt. 1901. A significantly lower incidence of the precore stop codon was found in anti-HBe–positive serum samples from Haikou (17.6%), whereas in other areas the percentages of this mutation in anti-HBe positive sera ranged from 47.4% to 78.9%. In HBeAg-positive samples, the rate of e-minus mutant in coexistence with wild-type virus was low in specimens from Haikou (9.5%) and Changchun (2.9%) compared to other areas in China. In contrast, coexistence of mutant and wild-type virus was frequently detected in samples from Wuhan (50.0%). ***J Med Virol 51:85–89, 1997.***

KEY WORDS: HBV; e-minus variants; precore mutation

INTRODUCTION

Hepatitis B viruses (HBV) with mutations in the precore region leading to defects in secretion of hepatitis B "e" antigen have been found worldwide. However, the incidence of this e-minus variant is more common in Mediterranean and Far East populations [Brown et al., 1992]. In a previous study of e-minus HBV strains in China, we have detected ten types of precore variants with mutations in the nucleotide sequences of the precore region [Wei et al., 1994]. Among the variants, six types had either only a TGG to TAG mutation at precore codon 28 (A28 mutant) or the A28 mutation in combination with other mutations. One variant had a ATG to CTG mutation at the initiation codon, and the remainder of the sequence did not have changes which would hamper the secretion of HBeAg [Wei et al., 1994]. It has been reported that patients infected with e-minus mutants have a prolonged time course and more severe form of hepatitis [Yokosuka and Bonino, 1994; Omata, et al., 1991]. However, other reports found e-minus variants in patients irrespective of their clinical status, ethnic origin, or subtypes of HBV [Turkaspa et al., 1992]. In a recent review by Hadziyannis [1995], the epidemiology, clinical manifestations, pathogenesis, and response to treatment of HBeAg-negative chronic hepatitis B have been fully presented and discussed. Since China is a country of very marked diversity with a large population of HBV-infected patients, we decided to investigate the frequency of the A28 precore variant in 446 serum samples from five distinct areas of the country.

MATERIALS AND METHODS

Serum Samples

Four hundred forty-six serum samples from adult HBsAg-positive chronic hepatitis B patients, with a protracted clinical course from 6 months to 6 years, were collected, following ethnic guidelines, from hospitals of the following five cities: Shanghai (65 samples), Chengdu (97 samples), Wuhan (100 samples), Haikou (94 samples), and Changchun (90 samples). Sera were

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kept frozen before assay for e-minus mutants. HBeAg and anti-HBe were assayed by EIA kits (Abbott Laboratories, North Chicago, IL).

DNA Extraction and Amplification

Each sample of serum (0.1 ml) was treated with Proteinase K (300 μg/ml) in 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 0.1 mol/L NaCl for 2 hours at 60°C. After phenol, chloroform, and isoamyl alcohol extraction, DNA was precipitated with ethanol and resuspended in 30 μl of sterile distilled water. Polymerase chain reaction (PCR) was performed to amplify the precore and part of the core region using the following primers: (a) 5′-GCTCTAGGCATAAATTGGTCTG-3′ (sense, nt 1785–1806), (b) 5′-CTTGAACATAGCTGACTACTAATTC-3′ (antisense, nt 2148–2172 nt). Two microliters of serum DNA, 1 μl of each primer (35 μmol/L), and 1 μl of each dNTP (10 mmol/L) were added to the reaction buffer followed by 2.5 units of Tqa DNA polymerase (Boehringer-Mannheim) in a total volume of 50 μl. The amplification reaction consisted of 35 cycles of 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C. After 35 cycles, an additional extension step was performed at 72°C for 10 minutes. Positive and negative serum controls and water controls were included in each PCR experiment. The PCR product was analysed by 1.2% agarose gel electrophoresis with ethidium bromide, and a 391-bp band corresponded to the predicted size of the amplified DNA. The sensitivity of this PCR assay followed by dot-blot hybridization could detect 10^{-5} pg of HBV DNA in the original sample.

Labelling of Oligonucleotide Probes

As reported previously [Li et al., 1990], three oligo probes were used: M0 (wild-type nt 1887–1908 5′-TGGGTGGCTTTGGGGCATGGAC-3′), M1 (with one point mutation at nt 1898 5′-GGGTGGCTTTAGGGCATGGAC-3′), and M2 (with two point mutations at nt 1898 and nt 1901 5′-TGGGTGGCTTTAGGACATGGAC-3′). The oligonucleotides were individually labelled with α-^{32}P-dCTP (10 mCi/ml) using a DNA 3′ end-labeling kit (Boerhinger-Mannheim).

Dot Hybridization With Oligoprobes

According to a slightly modified method of Li et al. [1990], eight μl of the PCR product was mixed with an equal volume of of 20× SSC, denatured at 94°C for 4 minutes, and dotted onto positively charged nylon filter membranes (Boerhinger-Mannheim). The membrane was denatured with 1.5 NaCl, 0.5 M NaOH for 5 minutes and neutralised with 1.5 M NaCl 0.5 M Tris-HCl (pH 7.2), 1 mM EDTA, for 1 minute. The filter was then air-dried and subsequently baked at 120°C for 30 minutes. Purified DNAs from plasmids harbouring either the wild-type precore sequence, the M1 precore mutant, or the M2 precore mutant were included as controls to confirm probe specificity.

Filters were prehybridised for 2 hours and subsequently hybridised for 2 hours with a solution containing 6× SSC, 10× Deinhardt solution, 0.2% sodium dodecyl sulfate (SDS), 100 μg/ml sheared salmon DNA, and 2×10^6 cpm/ml of the oligonucleotide probe. For convenience, aliquots of each of PCR reaction were spotted on duplicate filters, and one filter was hybridised with the wild-type (M0) oligonucleotide probe, while the other was hybridised with a mixture of the mutant probes (M1 + M2). After hybridisation, filters were washed successively once in 6× SSC/0.1% SDS for 5 minutes, 2× SSC/0.1% SDS for 15 minutes, and finally in 0.5× SSC/0.1% SDS for 5 minutes. The temperature for washing was 67°C for M0 and 63°C for M1 + M2. The filters were exposed to X-ray film at −70°C for 3 hours and developed. Specificity of M0 and M1, M2 probes was confirmed by showing no cross-hybridization among filters with M0 and M1 + M2. The filters were exposed to X-ray film at −70°C for 3 hours and developed. Specificity of M0 and M1, M2 probes was confirmed by showing no cross-hybridization among filters with M0 and M1 + M2 DNA.

TABLE I. Precore Region Detected by PCR and DNA Hybridization†

	Serum HBeAg+	Serum anti-HBe+
Shanghai	55.0% (11/20)[a]	42.2% (19/45)
Chengdu	54.0% (27/50)	25.5% (12/47)
Wuhan	47.4% (18/38)	30.6% (19/62)
Haikou	75.0% (42/56)*	44.7% (17/38)
Changchun	87.5% (69/83)*	42.9% (3/7)

†Sensitivity of this assay was 10^{-5} pg of HBV DNA.
[a]Positive percentage (No. of positive samples/No. of samples assayed).
*Compared to Wuhan samples, $P < .01$.

PCR and Direct Sequencing of Samples From Wuhan, Changchun, and Haikou

To study the possible mechanisms involved in the low incidence of A28 precore mutation detected in Haikou, 36 PCR products amplified by primer seq1: 5′-CCCAAGGTCTTACATAAGAGG-3′ (sense, nt 1643–1663) and seq2: 5′-GGTGGCCAGATTCATCAACT-3′ (antisense, nt 2112–2093) were sequenced. Nine sera were from Wuhan, 11 sera were from Changchun (14 HBeAg positive, six anti-HBe positive), and 16 sera were from Haikou (five HBeAg positive, 11 anti-HBe positive). PCR products were purified using Qiagen PCR purification kit, and 50 ng of purified products was sequenced by the dideoxy chain termination method, using a 5′ end-labelled (γ-^{32}P-ATP) Seq1 primer fmol sequencing kit (Promega).

RESULTS

HBV Precore Gene Fragment Detected in Anti-HBe–Positive and HBeAg–Positive Serum Samples

Among the 446 serum samples studied, 247 were HBeAg positive and 199 were positive for anti-HBe. In the HBeAg-positive group, precore fragment was successfully amplified by PCR and confirmed by dot-blot hybridization in 167 samples (67.6%). As shown in Table I, the positive percentage of precore gene fragment de-

TABLE II. A28 Variants Detected in Anti-HBe–Positive Samples

	Only M0	Only M1/M2	M0 + M1/M2	Total M1/M2
Shanghai	47.4% (9/19)[a]	10.5% (2/19)	36.8% (7/19)	47.4% (9/19)[b]
Chengdu	8.3% (1/12)	25.0% (3/12)	50.0% (6/12)	75.0% (9/12)[c]
Wuhan	21.0% (4/19)	36.8% (7/19)	42.1% (8/19)	78.9% (15/19)
Haikou	82.4% (14/17)	5.9% (1/17)	11.8% (2/17)	17.6% (3/17)*
Changchun	(1/3)	(1/3)	(1/3)	(2/3)

[a]No. of positive samples/total no. of samples assayed.
[b]One sample was negative with M0 and M1/M2. Insufficient serum for cloning and sequencing.
[c]Two samples were negative with M0 and M1/M2. Insufficient serum for cloning and sequencing.
*Compared with Wuhan samples, $P < .01$; compared with Chengdu samples, $P < .01$.

tected varies among samples collected from different areas. Higher percentages were found in samples from Haikou and Changchun, whereas lower percentages were found in Wuhan, Chengdu, and Shanghai samples. Compared to Wuhan samples, incidence of precore positive samples was significantly higher in Haikou ($P < .01$) and in Changchun ($P < .01$). Among the anti-HBe samples studied, 70 samples were positive for precore gene fragment, revealing that overall in 35.2% (70/199) of anti-HBe–positive patients replication of HBV still persists. In contrast to the HBeAg-positive samples, percentages of replication HBV in anti-HBe–positive samples showed no statistical differences in all five geographic areas (Table I).

A28 Precore Mutation in Anti-HBe–Positive Serum Samples

Since only seven anti-HBe–positive samples were obtained from Changchun, the A28 mutation rate was compared only among the other four cities (Table II). A significantly lower A28 mutation rate was found in Haikou samples (17.6%) when compared to samples from Wuhan (78.9%, $P < .01$) or to samples from Chengdu (75.0%, $P < .01$). In anti-HBe–positive serum samples collected from all areas, the percentages of coexistence of wild-type virus and A28 mutant were usually higher than those of samples only infected with A28 mutant (Table II).

A28 Precore Mutation Detected in HBeAg-Positive Serum Samples

Among 247 HBeAg-positive serum samples, only four samples hybridised exclusively with the M1/M2 oligo probes, indicating that either A28 mutants were exclusively present in these four samples or A28 mutants were the predominant form of HBV in these patients. Comparing samples from four cities, the percentage of coexistence of wild-type virus and A28 mutant was higher (50%) in samples from Wuhan than those from other cities (Table III).

Precore Sequences of Samples From Wuhan, Changchun, and Haikou

The sequence of codon 28 from all 36 samples from Wuhan, Changchun, and Haikou correlated with the dot-blot hybridization assay using M0 and M1 + M2 probes. Among the 16 samples from Haikou, seven were CCC sequence in codon 15, and none of these samples had A28 mutation; in eight samples with A28 mutation, the sequence of codon 15 was CCT. One sample did not show any mutation, either in codon 15 or in codon 28. Among the 20 samples from Wuhan and Changchun, the sequence of codon 15 was consistently CCT. In addition, point mutations were found in the core promoter region from Haikou samples (data not shown).

DISCUSSION

Hadziyannis [1981] was the first to introduce the term "anti-HBe–positive—chronic active hepatitis," and since then, continuous HBV replication has also been found in Oriental and African patients despite sero-conversion to anti-HBe. In Greece and Italy, e-minus variants are common, whereas in British patients, chronic hepatitis B is rarely associated with e-minus variants [Carman et al., 1991]. In a previous study of 20 anti-HBe–positive Chinese chronic hepatitis B patients, using the complete viral genome as the probe for HBV DNA hybridisation, 15% of these patients were serum HBV DNA positive, while 65% were HBV DNA positive when liver tissue was studied by southern blot and DNA hybridisation [Wen et al., 1986]. In this study, using PCR and dot-blot hybridisation assay which could detect as little as 10^{-5} pg HBV DNA, 35.2% of 199 anti-HBe–positive serum samples from five different cities in China were found to have circulating HBV precore gene fragment. It would be expected that a higher percentage of HBV DNA could be detected if liver tissues were examined or if other regions of HBV genome (envelope or polymerase regions) were included. Worldwide HBeAg-negative chronic hepatitis B has been summarized as from 7 to 30% [Hadziyannis, 1995]. From our data, a conservative estimate is that in at least approximately 30% of Chinese anti-HBe–positive patients, active HBV replication still persists.

To study whether there are differences in frequencies of HBV e-minus strains in various geographic regions in China, all 446 samples were collected from the Han ethnic group, and all were from adult chronic hepatitis B patients who were clinically diagnosed as chronic hepatitis B with a history of viral hepatitis for at least 6 months. Data indicate that serum samples from Haikou and Changchun have higher percentages of precore gene fragment in HBeAg-positive sera. In addition, in anti-HBe–positive sera from Haikou, a significantly low

TABLE III. A28 Variants Detected in HBeAg-Positive Samples

	Only M0	Only M1/M2	M0 + M1/M2	Total M1/M2
Shanghai	63.3% (7/11)[a]	0.0 (0/11)	27.2% (3/11)	27.2% (3/11)[b]
Chengdu	81.5% (22/27)	3.7% (1/27)	14.8% (4/27)	18.5% (5/27)
Wuhan	50.0% (9/18)	0.0 (0/18)	50.0% (9/18)	50.0% (9/18)*
Haikou	90.5% (38/42)	4.7% (2/42)	4.7% (2/42)	9.5% (4/42)
Changchun	97.1% (67/69)	1.4% (1/69)	1.4% (1/69)	2.9% (2/69)

[a]No. of positive samples/No. of samples assayed.
[b]One sample was negative with M0 and M1 + M2. In sufficient serum for sequencing.
*Compared with Haikou samples, $P < .01$; compared with Changchun samples, $P < .01$; compared with Chengdu, $P < .05$.

incidence of e-minus variants (17.6%) was observed. In a study among Chinese hepatitis B patients in Hong Kong (also in the southern part of China), of 62 HBeAg-negative patients, 26 (42%) were infected by the wild-type virus, while 36 (58%) were infected with mutants in the precore region [Lok et al., 1994]. This observation is similar to the frequency of e-minus variants in Shanghai and Chengdu but distinct from that of Haikou. Since Haikou city is on an island while Hong Kong is located in the mainland, circulating HBV strains in these two south China cities could differ. Sequence analysis of the precore regions indicates that the sequence of codon 15 was CCC in seven out of 16 samples from Haikou, whereas codon 15 was CCT in all 20 samples from Wuhan and Changchun. Since the sequence of codon 15 was found to be related to the G to A mutation at A28 due to the stability of secondary structure of encapsidation signal [Li et al., 1993; Lok et al., 1994], the higher percentage of CCC at codon 15 could account for the lower incidence of A28 variant detected in Haikou. It is also noted that the coexistence of wild-type virus and the e-minus variants was low in HBeAg-positive samples from Changchun (1.4%) and Haikou (4.7%), whereas in Wuhan samples, the coexistence was as high as 50% ($P < .01$).

Numerous factors could be involved in the frequency of emergence of the A28 precore variant. These factors include age at the time of infection, the duration of infection, sex, coinfection with other viruses, immune responses of the host, host genetic factors, and the biological as well as molecular biological characteristics of the viral strains. The low incidence of the A28 variant in Haikou and its association with the nucleotide sequence of codon 15 suggests a role for the genetic heterogeneity of infecting HBV strains. Besides, one may presume that HBV strains in Wuhan could be more prone to mutation in the precore region, and thus would occur more frequently and appear earlier during the course of hepatitis B infection, even prior to seroconversion to anti-HBe. It is also possible that HBV strains in Haikou and Changchun could replicate at a higher level but with less propensity to develop precore stop codon mutation. By comparing the precore A28 variants detected in different geographic areas, we have presented evidence that the precore sequence differs in HBV strains prevalent in different areas in China. In a sero-epidemiological survey carried out in the early 1980s, the prevalence of HBsAg was found to be lower in northern China and the northeastern provinces (5–9%), but higher in the south and southeastern provinces (10–16%) [Jiang, 1981]. Since blood transfusion, usage of syringe and needles, as well as acupuncture applications were employed similarly throughout the country, this difference could be related to the biological characteristics, including the infectivity of different HBV strains. Further studies are necessary to evaluate whether local differences in HBV strains may be responsible for the observed prevalence of development of precore codon mutations.

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